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(54) Title: MATERIAL AND METHOD FOR PROMOTING GROWTH OF ANAEROBIC BACTERIA

(57) Abstract

A material and method for promoting the growth of anaerobic bacteria which includes a nutrient media containing a hydrogen donor and sterile membrane fragments of mitochondria having an electron transfer system which reduces oxygen to water. Dissolved oxygen in the medium is removed by adding the sterile membrane fragments to the nutrient medium and holding the medium at a temperature of about 10°C to about 60°C until the dissolved oxygen is removed.

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MATERIAL AND METHOD FOR PROMOTING GROWTH OF ANAEROBIC BACTERIA

Field of the Invention

The present invention relates generally to promoting the growth of anaerobic bacteria and in particular relates to the use of sterilized mitochondrial derivatives for the production of anaerobic conditions in media to promote the

Many of the bacteria present in natural environments are sensitive to oxygen and will not grow in its presence. When these organisms (known as anaerobes) are brought into the laboratory, it is often necessary to employ cumbersome physical and chemical techniques in order to get them to grow. Some of these bacteria produce diseases of man and related species. Others produce important industrial end products such as methane, hydrogen and various alcohols. The manipulation of these organisms is, to some degree, limited by the ease with which they can be grown.

In order to grow anaerobes, it is necessary for the medium in which the anaerobe is to be grown to be substantially free of oxygen. Oxygen can be removed fairly efficiently from such media by sparging with high purity nitrogen or other inert gas. However, these liquid media are subject to foaming so that this process presents a number of mechanical difficulties. Further, after sparging is stopped, the medium is easily recontaminated with oxygen.

Oxygen can also be removed from liquid media by the addition of reducing agents. However, most of these agents are strong reducing agents and any residual agent or its by-products in the media tends to inhibit the subsequent growth of anaerobes in the media. Also, the reducing agent which is consumed during the initial removal of oxygen is not available to act upon oxygen which might later find its way into the system.

In the case of a solid medium, such as agar, oxygen

removal with an inert gas is difficult to accomplish because the oxygen in the medium comes to equilibrium with the inert atmosphere slowly and never reaches a zero concentration. Reducing agents can be added to a solid media but again there is still the problem of the inhibition of anaerobic growth in the media due to any residual reducing agent or its by-products as well as the problem of providing the system with the ability to consume any oxygen which might later find its way into the system.

United States Patent 4,476,224, issued October 9, 1984, to Howard I. Adler, discloses that sterilized membrane fragments from certain oxygen-consuming bacteria may be incorporated into media to remove oxygen rapidly and completely from the media. This patent teaches that use of these membrane fragments overcomes many of the problems recited above.

The principle object of this invention is to provide a material with similar oxygen-consuming properties, but derived from a distinctly different, non-bacterial source. More specifically, this invention provides oxygen consuming enzyme systems from mitochandria obtained from a variety of higher non-bacterial organisms, as well as a method for using such systems to grow anaerobic bacteria. The oxygen-consuming enzyme systems of this invention, obtained from mitochandria exhibit properties which make them useful for increasing the range of anaerobic bacteria that can be grown using the membrane fragments obtained from bacteria disclosed in U.S. 4,476,224. Other objects and advantages of the invention will become apparent from the following descriptions of various embodiments and examples of the invention.

In accordance with this invention, when membrane fragments from oxygen-consuming mitochondria are

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incorporated into media, either liquid or solid, oxygen is rapidly and completely removed from the media. As will be pointed out, in some instances it is necessary to incorporate in the media a small amount of a suitable hydrogen donor so that reduction of the oxygen to water is facilitated. The sterile membrane fragments of this invention can be employed without foaming problems. They are non-toxic and the presence of these membranes has no adverse effect upon the growth of the anaerobic bacteria in the media, even when used at high levels, such as a ten-fold excess. Further the presence of the membrane fragments in the media provides the media with the capacity to reduce additional oxygen which later may enter the system so that extreme methods of sealing the system are not required.

A great number of fungi, yeasts, and plants and animals have mitochondria that reduces oxygen to water, if a suitable hydrogen donor is present in the medium. Some of the sources of oxygen reducing membranes from these mitochondria are: beef heart muscle, potato tubers, spinach, Saccharomyces, Neurospora, Aspergillus, Euglena and Chlamydomonas. The process of producing the useful membrane fragments involves the following steps:

- 1. Yeast, fungal cells, algae and protozoa, having mitochondria membranes containing an electron transfer system which reduces oxygen to water can be isolated, are grown under suitable conditions of active aeration and a temperature which is conducive to the growth of the cells, usually about 20°C to 45°C in a broth media. Alternately, mitochondria may be obtained from cells of animal or plant origin.
- 2. The cells are collected by centrifugation or filtration, and are washed with distilled water.

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- 3. For the preparation of crude mitochondrial membrane fragments a concentrated suspension of the cells is treated to break up the cell walls and mitochondria. This is accomplished by known means, for example by ultrasonic treatment or by passing the suspension several times through a French pressure cell at 20,000 psi.
- 4. The cellular debris is removed by low speed centrifugation or by microfiltration (cross-flow filtration).
- 5. The supernatant or filtrate is subjected to high speed centrifugation (175,000Xg at 5° C) or ultrafiltration.
- 6. For the preparation of material of higher purity, the cells of step 2 are suspended in a buffer containing 1.0M sucrose and are treated by means which break up the cell walls or membranes but leave the mitochondria intact. This is accomplished by known means, for example, by ultrasonic treatment, passage through a French pressure cell at low pressure, enzymatic digestion or high speed blending with glass beads.
- 7. The cellular debris from step 6 is removed by differential centrifugation or filtration.
- 8. The supernatant or retentate from step 7 is passed through a French Press at 20,000 psi to break the mitochondria into small pieces.
- 9. Mitochondrial debris from step 7 is removed by centrifugation at 12,000Xg for approximately 15 minutes or by microfiltration.
- 10. The supernatant or filtrate from step 9 is subjected to high speed centrifugation (175,000Xg at 5°C) or ultrafiltration.
- 11. The pellet or retentate from step 5 (crude mitochondrial fragments) or the pellet or retentate

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from step 10 (purified mitochondrial membrane fragments) are resuspended in a buffer solution at a pH of about 7.0 to about 7.5. A preferred buffer solution is 0.02M solution of N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES).

12. The membrane fragments in the buffer solution are then passed under pressure through a filter having openings of a size which will retain any intact microorganisms to effect sterilization. Openings of about 0.2 microns are satisfactory.

13. The sterilized suspension is then preferably used promptly or stored for use at about -20°C or it may be freeze dried.

In use, a small amount of the sterile membrane fragment suspension is added to a liquid medium which is to be used for the growth of the anaerobic bacteria (about 25 to 3000 mp of fragments per liter of medium). The medium is permitted to stand for a short period of time at a temperature of from about 10°C to about 60°C until the oxygen is consumed. This action takes up to about 20 to 30 minutes, depending upon the concentration of the sterile membrane fragments and the temperature. At concentrations of about 500 mg/l and temperatures of about 35°C, removal is effected in from about 2-8 minutes. After the oxygen is removed, an inoculum of anaerobic bacteria is introduced into the medium. The inoculated medium is then incubated for the growth period at the proper temperature for the bacteria which are to be grown. Preferably, the air space above the liquid medium in its container is flooded with an inert gas such as nitrogen. This minimizes the amount of oxygen that must be removed by the membrane system and prolongs the life of the oxygen-consuming system. also gives assurance that, if there is an accidental leak of air into the system, the system will consume that air

and insure that the growth of the anaerobic bacteria will

In the case of a solid medium, such as agar, the .bebratar ad for

may leak into the dish. consuming reasonable amounts of oxygen from any air which precedition since the membrane system is capable of obtained on rapidly growing anaerobes without such a inert gas, such as nitrogen, but good results can be To evergeomts as at benistaries of Videveleve bluode seatsib inter proper temperature for growth. Agent record then incculated with the anaerobe to be grown and incubated ai muibem edT .evods tuo betrioq es ,etrempsat ensadaes. alivate and to noits transcooping any temperature of less than 20 to 30 minutes. The time of removal depends until the oxygen is consumed, usually in a period of time held at a temperature of from about 10°C to about 60°C at it hain into Petri dishes, or the like, in which it is .muibem to retil req etremgart to gm 0002 of at mutbem edT molten state at approximately 45°C at a level of about 25 s ni muibam ant ot babbs yidsnafang ai noitsnagang ansndmam

anserobic bacteria. Suitable hydrogen donors are sodium which does not interfere with the growth of the selected be necessary to add a small amount of a hydrogen donor In the event that a synthetic media is employed, it may

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Some 18 species of anaerobic bacteria representing 8

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penetrate the cell walls the system does not adversely affect the anaerobes being grown. This is in contrast to a system in which a chemical reducing agent is used to accomplish the removal of dissolved oxygen when the residual reducing agent or its by-product may penetrate the cell walls. Thus, because the anaerobic bacteria are being grown under more natural conditions, they flourish at a greater rate than in media which has been treated with such reducing agents.

EXAMPLE I

A nutrient broth is inoculated with <u>Saccharomyces</u>
<u>cervisiae</u> (ATCC 18790). The nutrient broth employed is
Malt Extract Broth sold by Difco Laboratories, Detroit,
Michigan. The inoculated broth is maintained at 24°C and
is actively aerated. The growth is continued until it is
in the late logarithmic phase.

The broth containing the <u>Saccharomyces cervisiae</u> cells is centrifuged at 4,000Xg to harvest the yeast cells. The cells are washed with distilled water and centrifuged. This washing and centrifuging are repeated.

The harvested cells are cooled to 0-2 °C and suspended in two volumes of a buffer containing 1.0M sucrose, 0.02M tris-(hydroxymethyl)amino methane (Tris) and 0.0001 sodium ethylene diamine tetra acetate (EDTA) at pH 7.4. To this suspension is added an equal volume of glass beads (average diameter 0.15mm) and the mixture is homogenized at 15,000 RPM in a steel blender for 3-4 minutes. The glass beads are allowed to settle and the supernatant is decanted.

The supernatant is centrifuged at 2400Xg for 20 minutes to remove unbroken cells and debris. The mitochondria are harvested from the supernatant by centrifuging at 20,000Xg for 15 minutes.

The mitochondria are then resuspended in 0.02M HEPES buffer at pH 7.5 (CalBiochem-Behring Corporation, LaJolla, California) and passed three times through a French pressure cell at 20,000 psi.

The suspension of the membrane fragments in the buffer solution is then passed through a 0.22 micron filter under pressure to produce the sterile membrane fragments to be used to produce anaerobics in the media to be used for growing anaerobes. The suspension of membrane fragments is stored at about -20°C. The dry weight of the solids in the suspension is about 30 mg/ml which is determined by desiccating samples over phosphorous pentoxide in a vacuum.

EXAMPLE II

Ten microliters of the suspension of sterilized membrane fragments from Example I is added per milliliter of oxygen saturated Difco Nutrient Broth at 37°C. In five minutes all of the oxygen is removed. A portion of the nutrient broth, treated with the sterilized membrane fragments and held until anaerobic conditions are obtained, is inoculated with Clostridium difficile and incubated at 37°C for 16 hours in a sealed container. A luxuriant growth (approximately 10/9 cells per ml) is observed.

EXAMPLE III

Ten microliters of the sterile membrane fragment preparation of Example I is added per milliliter of synthetic medium which is supplemented by the addition of sodium lactate to a final concentration of 0.25M. The synthetic medium consists generally of inorganic salts, ethanol and sodium acetate. It is prepared from reagent grade chemicals. A sample of medium containing the membrane fragments is held at 37°C to remove oxygen and

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is inoculated with <u>Clostridium kluyveri</u>. The inoculated medium is incubated at 34°C for 96 hours. At the end of that time a luxuriant growth (approximately 10/9 cells per ml) is observed.

EXAMPLE IV

The sterile membrane fragment suspension of Example I is added to molten agar at 45°C at a level of 10 microliters of suspension per milliliter of agar. The agar is poured into a Petri dish and held to obtain anaerobic conditions. It is inoculated with <u>Clostridium kluyveri</u> and incubated in the presence of an inert gas atmosphere at a temperature of 34°C for a period of 96 hours. Colonies with a diameter of approximately 2 millimeters are observed at that time.

Similar results are observed with sterilized membrane fragments from beef heart muscle, potato tubers, spinach, Saccharomyces, Neurospora, Aspergillus, Euglena and Chlamydomonas. In all cases the concentration of membrane fragments in the sterile membrane suspension is about 25 - 30 mg/ml. At 37°C all of the oxygen is removed from the nutrient media in from about 2 to about 8 minutes when the membrane fragment suspension is present at from about 10 to about 100 milliliters per liter of broth (250-3000 mg of fragments per liter of broth). At higher levels, oxygen removal is more rapid than at lower levels.

As pointed out above, when a synthetic media or one not containing a hydrogen donor is employed, a hydrogen donor which is compatible with the bacteria being cultured should be employed to supplement the medium. The hydrogen donor, e.g. sodium lactate, sodium succinate, alpha-glycerol phosphate, or sodium formate should be employed at a level of the order of 0.15 to 0.25M.

Anaerobic bacteria which can be successfully grown in nutrient media treated by the sterile membrane system described herein are:

Clostridium difficile Clostridium tetani Clostridium kluyveri Clostridium sporogenes Clostridium perfringens Clostridium sordelli Clostridium butyricum Clostridium bifermentans Clostridium acetobutyricum Peptostreptococcus anaerobius Peptostreptococcus micros Peptostreptococcus magnus Desulfovibrio vulgaris. Fusobacterium nucleatum <u>Viellonelia</u> parvula Propionibacterium acnes Eubacterium limosum Racteriodes fragilis

The use of sterile membrane-containing media may be used in clinical laboratories to stimulate growth of anaerobic bacteria from human patients. Sterile membrane containing media may be used to increase the survival of anaerobes in medium used to transport samples from the patient to the laboratory and also for the determination of antibiotic sensitivity patterns of anaerobic bacteria. It also has use in producing the anaerobic conditions required in many industrial fermentation processes. The use of sterile membrane fragments, as described above, produce little or no toxic side effects when used in amounts much greater than those required to achieve oxygen-free

conditions. Small quantities of the membrane fragments reduce a medium that is initially saturated with oxygen to an anaerobic condition and maintain that condition even though small amounts of air are introduced.

Various features of the invention are set forth in the appended claims.

What is claimed is:

- 1. A nutrient medium for growing anaerobic bacteria which includes a hydrogen donor and sterile membrane fragments derived from mitochondria having membranes containing an electron transfer system which reduces oxygen to water.
- 2. The nutrient medium of claim 1 in which the sterile membrane fragments are present in a concentration of from about 25 to 3000 mg/l.
- 3. A nutrient medium for growing anaerobic bacteria which includes a hydrogen donor and sterile membrane fragments derived from yeast, fungi, plants, and animals selected from the class consisting of beef heart muscle, potato tubers, spinach, Saccharomyces, Neurospora, Aspergillus, Euglena and Chlamydomonas.
- 4. The nutrient medium of claim 3 wherein the sterile membrane fragments are derived from yeast and fungi selected from the class consisting of beef heart muscle, potato tubers, spinach, <u>Saccharomyces cervisiae</u>, and <u>Aspergillus cryzae</u>.
- 5. The nutrient medium of claim 1 wherein the hydrogen donor is selected from the group consisting of sodium lactate, sodium succinate, alpha-ketoglutarate, sodium formate and alpha-glycerol phosphate.

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- 6. The method of removing dissolved oxygen from a nutrient medium for anaerobic bacteria comprising the steps of introducing sterile membrane fragments derived from mitochondrial membranes which contain an electron transfer system which reduces oxygen to water in the presence of a hydrogen donor to the nutrient medium and maintaining the medium containing sterile membrane fragments at a temperature of from about 10°C to about 60°C until the dissolved oxygen is converted to water.
- 7. The method of claim 6 further comprising the step of adding a hydrogen donor to the nutrient medium.
- 8. The method of claim 7 wherein the hydrogen donor is selected form the group consisting of sodium lactate, sodium succinate, alpha-ketoglutarate, sodium formate, and alpha-glycerol phosphate.
- 9. A method of removing dissolved oxygen from a nutrient medium for anaerobic bacteria comprising the steps of introducing sterile membrane fragments, derived from yeasts, fungi, plants and animals selected form the class consisting of beef heart muscle, potato tubers, spinach, Saccharomyces, Neurospora, Aspergillus, Euglena and Chlamydomonas, to the medium and maintaining the medium containing sterile membrane fragments at a temperature of from about 10°C to about 60°C until the dissolved oxygen is converted to water.
- 10. The method of claim 9 wherein the sterile membrane fragments are derived from yeast and fungi selected from the class consisting of beef heart muscle, potato tubers, spinach, Saccharomyces cervisiae, and Aspergillus oryzae.

INTERNATIONAL SEARCH REPORT

International Application NoPCT/US87/03290

According to International Patent Classification (JPC (4): C12N 1/20, 1/00 U.S. C1: 435/253,262 Iffeld StarkCatton System	According to International Pattern Classification (PC) of 10 cells Nanocal Classification and IPC IPC(4): C12N 1/20, 1/00 IL.S.C.I: 435/253,262 Minimum Documentation Searches	The comment of the process of cited documents: 12 The comment of the process of comment defining the general state of the art which is not considered to be of particular relevant to fast and page 533 and page 534 and 539. Accoument defining the general state of the art which is not considered to be of particular relevant to published on or after the international classificator relevance in the cited to relevant to the comment of the pages 533 and page 534 and 539. Accoument defining the general state of the art which is not considered to be of particular relevant to subtless the publication of controller to the state in the pages 533 and page 534 and 539. Accoument defining the general state of the art which is not considered to be of particular relevance to the considered to be of particular relevance to the considered of controller state in the publication date of another citation or other social reason (test pages to the more than the publication date of another citation or other social reason (test pages to the pages 533 and page 534 and 539. Accoument defining the general state of the art which is not considered to be of particular relevance to the considered or cannot be considered in the publication date of another citation or other special reason (test pages to the page to the page to cannot be considered for the page to the page of cannot be considered for the page of cannot be considered for cannot be considered for the confidence in the page of cannot be considered for cannot be considered for the page of cannot be considered for the confidence and the considered for cannot be considered for the considered for the considered for the cannot be considered for the cannot be considered for the cannot be considered for the considered for the cannot be considered for the cannot be considered fo	I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 3								
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